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TI Three human transforming genes are related to the viral ras oncogenes.
AU Shimizu K; Goldfarb M; Suard Y; Perucho M; Li Y; Kamata T; Feramisco J; Stavnezer E; Fogh J; Wigler M H
SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1983 Apr) 80 (8) 2112-6.

TI Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses.
AU Der C J; Krontiris T G; Cooper G M
SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1982 Jun) 79 (11) 3637-40.
FOR 103 claim 8

TI Identification and characterization of R-ras3: a novel member of the RAS gene family with a non-ubiquitous pattern of tissue distribution
AU Kimmelman, Alec; Tolkacheva, Tatyana; Lorenzi, Matthew V.; Osada, Masako; Chan, Andrew M. -L.
SO Oncogene (1997), 15(22), 2675-2685
103 for 6,7,9,10, 8??

TI The use of the polymerase chain reaction to map CD4+ T cell epitopes.
AU Nakagawa T Y; Von Grafenstein H; Sears J E; Williams J; Janeway C A; Flavell R A
SO EUROPEAN JOURNAL OF IMMUNOLOGY, (1991 Nov) 21 (11) 2851-5.

TI Immunoglobulin complementarity-determining region grafting by recombinant polymerase chain reaction to generate humanised monoclonal antibodies.
AU Lewis A P; Crowe J S
SO GENE, (1991 May 30) 101 (2) 297-302.
Nakagawa and lewis for adding restriciton sites onto pcr primers.

Thank you

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Gene, 101 (1991) 297–302

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Immunoglobulin complementarity-determining region grafting by recombinant polymerase chain reaction to generate humanised monoclonal antibodies

(Protein engineering; recombinant DNA; *Taq* polymerase; nucleotide sequencing; CAMPATH-1H; digoxin)

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SUMMARY

We describe an approach to rapidly generate humanised monoclonal antibodies by grafting rodent complementarity-determining regions onto human immunoglobulin frameworks using recombinant polymerase chain reaction (PCR) methodology. The approach was applied to grafting a rat complementarity-determining region onto a human framework and amplifying the entire humanised heavy chain. The terminal oligodeoxyribonucleotide primers incorporated restriction sites to allow forced cloning into plasmid vectors for sequencing and expression. No nucleotide errors were introduced into the 1463-bp sequence even after sequential applications of PCR.

INTRODUCTION

The use of rodent mAbs for human therapy and in vivo diagnosis has been hampered by their immunogenicity in man (Miller et al., 1983; Jaffers et al., 1986). The ensuing anti-globulin response can reduce the circulating half life of the rodent mAbs and cause immune complex hypersensitivity. To avoid this scenario human mAbs would be preferred. Technical difficulties, however, render conventional hybridoma technology inappropriate for the generation of most human mAbs (Larrick et al., 1985).

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Abbreviations: aa, amino acid(s); bp, base pair(s); C, constant; CDR, complementarity-determining region; ds, double strand(ed); EtdBr, ethidium bromide; FR, framework region; H, heavy; Ig, immunoglobulin; kb, kilobase(s) or 1000 bp; mAb, monoclonal antibody; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PCR, polymerase chain reaction; ss, single strand(ed); V, variable; wt, wild type.

A new generation of mAbs for human therapy is being produced by protein engineering techniques. These mAbs are either chimeric, consisting of a human C region fused to a rodent V region (Morrison et al., 1984; Boulianne et al., 1985; Neuberger et al., 1985), or fully humanised, consisting of rodent CDRs grafted onto a human framework (Jones et al., 1986; Riechmann et al., 1988; Verhoeyen et al., 1988; Queen et al., 1989). Both these engineered constructs allow the class and sub-class of human Ig to be selected to generate the effector mechanisms of choice. However, as a significant antibody response is directed against the V region of murine antibody OKT3 (Jaffers et al., 1986), there may be an advantage for human therapeutic uses in fully humanised, CDR-grafted, constructs which have no rodent framework regions.

The generation of fully humanised antibodies has been performed to date using site-directed mutagenesis on ss DNA (Riechmann et al., 1988) or by constructing the whole V region using overlapping oligos incorporating the rodent CDRs on a human framework (Jones et al., 1986; Queen et al., 1989). Both of these techniques yield a low percentage

of correct product, for example using site-directed mutagenesis the overall yield of triple mutant in CAMPATH-1H heavy-chain humanisation was 5% (Riechmann et al., 1988). We describe here an application of recombinant PCR technology for the rapid generation of humanised mAbs.

EXPERIMENTAL AND DISCUSSION

(a) Recombinant PCR grafting of DX48 CDRH1 onto a human background

The objective of this experiment was to graft a CDRH1 from a rat anti-digoxin mAb (DX48) onto a human Ig backbone. The template used for the recombinant PCR was the previously humanised CAMPATH-1H heavy chain (Riechmann et al., 1988), a human IgG1 heavy chain with NEW (Saul et al., 1978) V region, which had been re-engineered from genomic into cDNA configuration, and had subsequently undergone site-directed mutagenesis to replace CAMPATH-1H CDRH2 and CDRH3 sequences with rat DX48 CDRH2 and CDRH3 yielding HUMDXCH.23 ss template in phage M13.

PCR reactions (Saiki et al., 1988) were carried out using ss HUMDXCH.23 template prepared as described in Sambrook et al. (1989). The reactions were performed in a programmable heating block (Hybaid) using 25 rounds of temperature cycling (94°C for 1 min, 50°C for 2 min, and

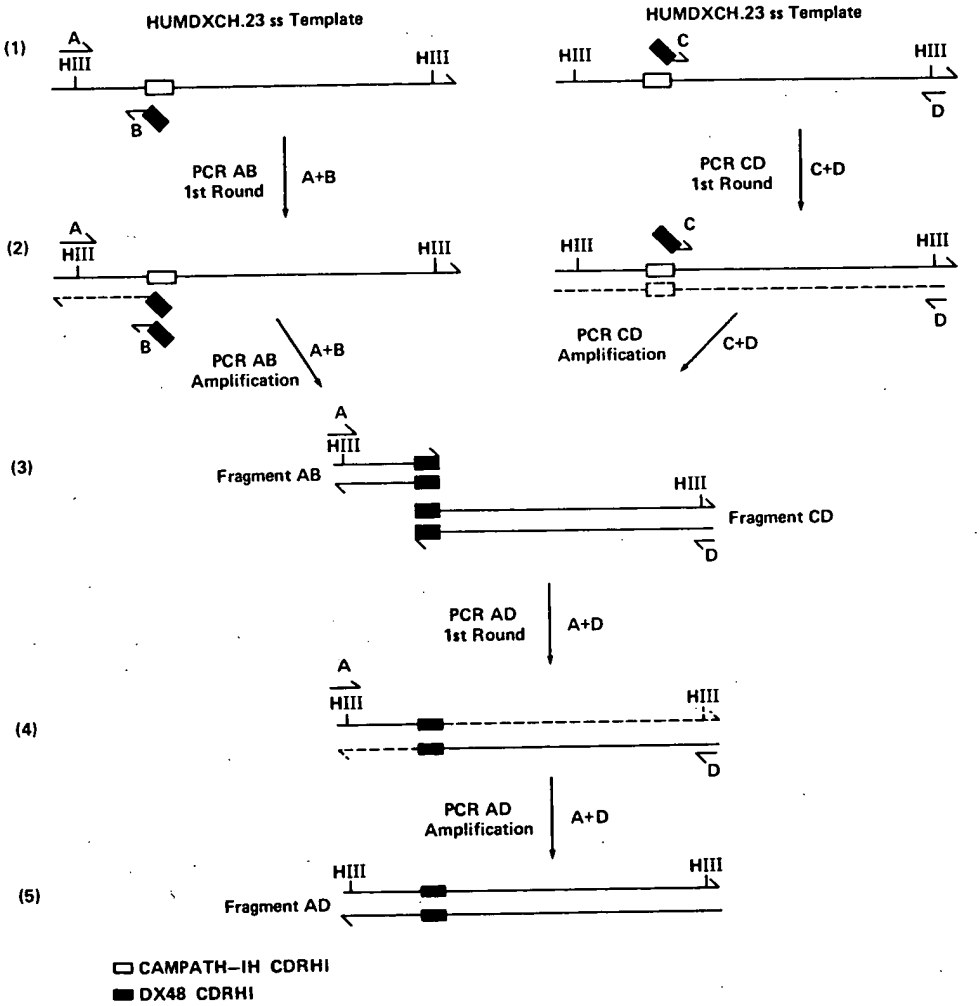

Fig. 1. Recombinant PCR strategy used to graft the DX48 CDRH1 onto the CAMPATH-1H framework. (1) The HUMDXCH.23 ss template is shown as a solid line, the *Hin*dIII sites at the termini of the insert are indicated by HIII. The CAMPATH-1H and DX48 CDRH1s are represented as open and blackened boxes, respectively. The 5′ → 3′ direction of each strand is shown by half arrowheads. Primers **A** + **B** and **C** + **D** are used in PCR reactions **AB** and **CD** to amplify fragments **AB** and **CD**, respectively. (2) The complementary strand of the ss template is synthesised in the first round of PCR (dashed line). (3) The amplified fragments **AB** and **CD** are shown with the homologous DX48 CDRH1 regions (introduced by oligos **B** and **C**) aligned. Fragments **AB** and **CD** are mixed and subjected to a recombinant PCR reaction, **AD**, with oligos **A** and **D**. The 3′ termini of the positive and negative strands of fragments **AB** and **CD**, respectively can anneal and act as primers for one another in the first round of PCR. (4) The annealed strands are extended to form the full-length recombinant product (the synthesised segments of DNA are shown as dashed lines) which is (5) amplified by primers **A** and **D** to produce the desired product, fragment **AD**.

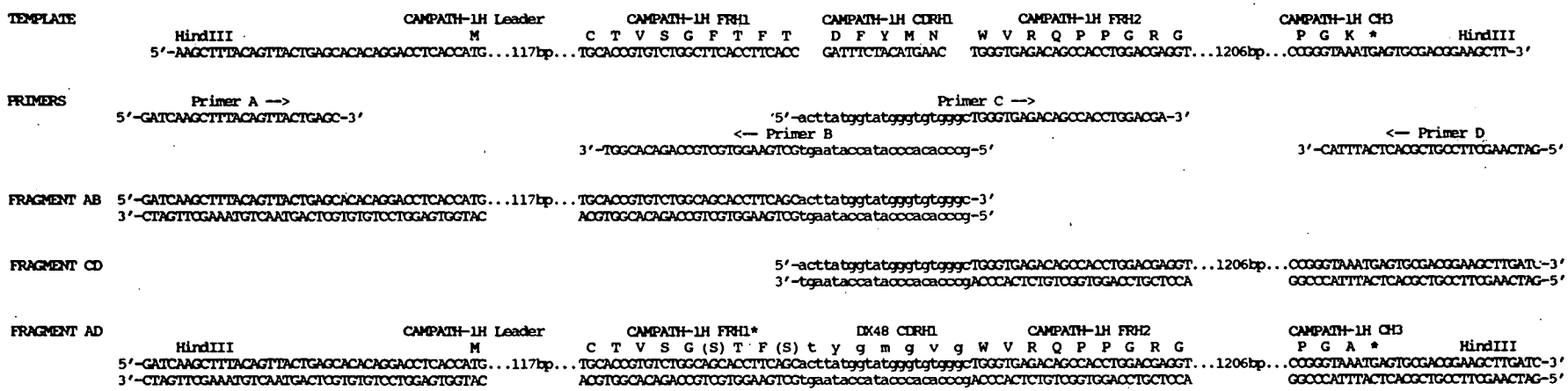

Fig. 2. Sequences of the HUMDXCH.23 template, oligo primers and PCR products. The nt sequence of three regions of the HUMDXCH.23 insert are shown in the top line incorporating: the first 42 bp at the 5′ end of the insert, including the start codon of the CAMPATH-1H leader sequence; the 3′ 27 bp of FRH1, the whole length of CDRH1, and the 5′ 27 bp of FRH2 from CAMPATH-1H; the final 27 bp at the 3′ terminus of the insert including the stop codon (asterisk) at the end of CAMPATH-1H CH3. The sequences are separated by 117 bp and 1206 bp, respectively. Primers **A** to **D** are shown aligned below the template sequence. Arrows indicate the direction in which each oligo can act as a primer. The sequence of the DX48 CDRH1 being transplanted is shown in lower-case letters. The ds PCR products, fragments **AB**, **CD** and **AD**, are shown aligned with the template and primers. The aa sequences of the template and fragment **AD** coding regions are shown above the corresponding nt sequences. Primer **B** possesses three point mutations reverting Phe^{27} and Thr^{30} of CAMPATH-1H FRH1 (Riechmann et al., 1988) to the Ser residues present in wt NEW FRH1. The altered FRH1 region of fragment **AD** is indicated by an asterisk (bottom, centre), the substituted aa of the sequence being in parentheses. The asterisks over TGA indicate stop codons.

72°C for 3 min) followed by a final 10-min step at 72°C. One μg of each primer, 50 ng of template and 2.5 units of *Taq* polymerase (Perkin–Elmer Cetus) were used in a final volume of 100 μl with the reaction buffer as recommended by the manufacturer. Synthetic oligos were made on a 7500 DNA Synthesizer (Milligen).

The approach used is summarised in Fig. 1. Two PCR reactions were carried out using the primer pairs **A** with **B**, and **C** with **D**, respectively. Primers **A** and **D** correspond to positive and negative strand oligos incorporating the *Hin*dIII sites at the 5′ and 3′ termini of the HUMDXCH.23 insert (Fig. 2). Primer **B** possesses negative strand sequence from the 3′ end of the CAMPATH-1H FRH1 region (with point mutations to convert Phe^{27} and Thr^{30} of CAMPATH-1H back to the Ser residues present in the NEW FRH1) together with the CDRH1 sequence of DX48 in place of the CAMPATH-1H CDRH1 (Fig. 2). Primer **C** is made up of the positive strand sequence of DX48 CDRH1, complementary to the CDRH1 region of primer **B**, running into the 5′ end of the CAMPATH-1H FRH2 (Fig. 2). In the first round of the **AB** and **CD** PCR reactions the HUMDXCH.23 a negative strand is synthesised from primers **B** and **D**, respectively (Fig. 1). In subsequent cycles fragments **AB** and **CD** are amplified (Figs. 1 and 2). The products of the two reactions thus constitute the whole length of the HUMDXCH.23 insert but with the point mutations stated above and the CAMPATH-1H CDRH1 replaced by the CDRH1 sequence of DX48. Fragments **AB** and **CD** both possess the DX48 CDRH1 sequence such that on denaturation and reannealing the overlapping sequences can anneal.

Excess primers were removed from the **AB** and **CD** PCR reactions by selective filtration on a Centricon 100 (Higuchi et al., 1988; Amicon). 50 μl of each reaction was placed into 2 ml of TE (10 mM Tris · HCl pH 8/0.1 mM EDTA) and mixed in the upper reservoir of the Centricon 100. The manufacturer's protocol was followed using a 25-min centrifugation in a fixed-angle rotor at 1000 × *g*, and the PCR products recovered in a 40-μl retentate.

Ten μl of the Centricon 100 retentate was subjected to a recombinant PCR reaction with primers **A** and **D** (Fig. 1) using the same conditions as performed in the primary PCR reactions above. The positive strand of fragment **AB** and the negative strand of **CD** contain the complementary DX48 CDRH1 sequences at their 3′ ends, and in the first PCR cycle can anneal and serve as primers for one another. Extension of the overlap produces the recombinant product fragment **AD** containing the transplanted DX48 CDRH1, and this is amplified by primers **A** and **D** in the subsequent rounds of PCR (Figs. 1 and 2). The remaining strands of fragments **AB** and **CD**, which are complementary at their 5′ ends, are not able to prime each other, but can act as templates for primers **A** and **D**. These generate more of the primary PCR products, although these fragments are not amplified in an exponential manner due to the absence of primers **B** and **C** in the reaction.

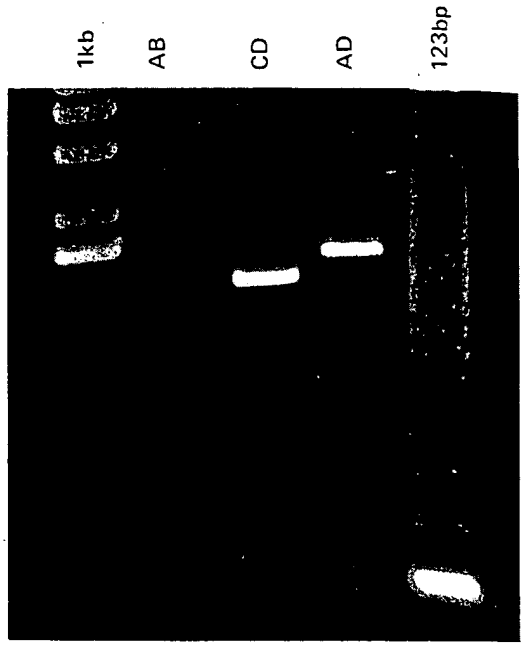

Fig. 3. Electrophoretic analysis of gel-purified PCR products. DNA fragments were analysed on a 0.8% agarose gel [0.8% type-II: medium EEO agarose (Sigma) in 89 mM Tris · borate/2 mM EDTA] and visualised by EtdBr staining. The expected sizes of the fragments are as follows: **AB**, 207 bp; **CD**, 1285 bp; **AD**, 1471 bp. The markers are 1-kb DNA ladder (BRL) and 123-bp DNA ladder (BRL).

Purified products of the PCR reactions **AB, CD** and **AD** described above are shown on an EtdBr-stained agarose gel in Fig. 3. The predominant band observed in each case was of the expected size, although additional minor bands also appeared in reaction **AD**.

(b) Cloning and sequencing of the recombinant PCR product

Fragment **AD** was gel-eluted, digested with *Hin*dIII (BRL) and cloned into the *Hin*dIII site of pUC-18 (BRL). The nt sequence of a clone containing the recombinant molecule was determined by plasmid priming following the dideoxy chain-termination method (Sanger et al., 1977) according to the Sequenase kit (USB) protocol. The entire 1463-nt insert was found to be of the correct sequence (data not shown), no misincorporations having resulted from the two sets of PCR reactions.

(c) Further applications of the technique to humanisation of immunoglobulins

The recombinant PCR technique should allow the generation of fully humanised mAb nt sequences using three rounds of PCR reactions (Fig. 4). Site-directed mutagenesis (Riechmann et al., 1988) and oligo gene synthesis

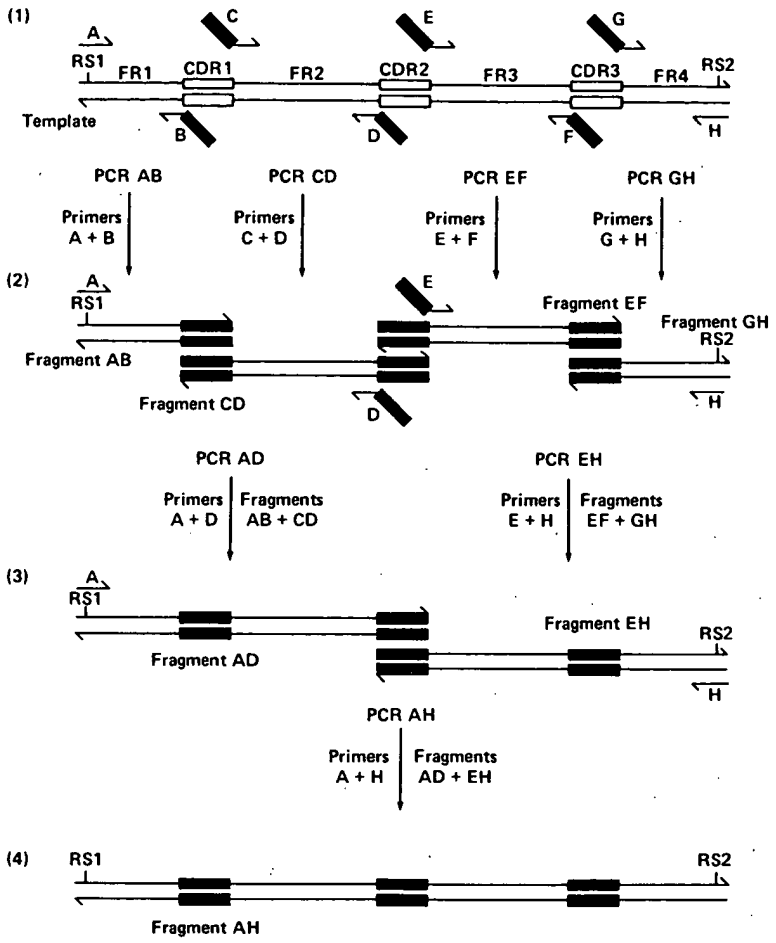

Fig. 4. Humanising a complete mAb chain by recombinant PCR. RS1 and RS2 denote restriction sites for any appropriate restriction endonuclease. The template is a human Ig sequence flanked by RS1 sites, from which the C region has been removed using an appropriate site RS2. Rodent and human CDR sequences are represented by blackened and open boxes, respectively, and the human FRs as solid lines. The 5′-3′ direction of each strand is shown by half arrowheads. (1) Four primary PCR reactions are performed using primer pairs **A + B**, **C + D**, **E + F** and **G + H** to generate (2) fragments **AB**, **CD**, **EF** and **GH**. Fragments **AB** with **CD**, and **EF** with **GH** are combined in recombinant PCR reactions **AD** and **EH** using primers **A + D**, and **E + H** respectively, thus producing (3) fragments **AD** and **EH**. These fragments are combined in a final recombinant PCR reaction **AH** using primers **A** and **H** to generate (4) the final recombinant product **AH**. This can be ligated into an RS1-cut vector with the human C region to produce the complete humanised mAb.

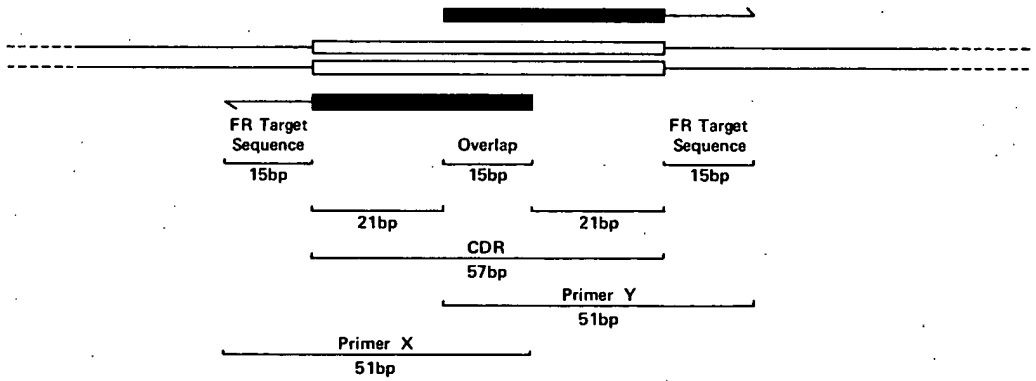

Fig. 5. Example of primers used to humanise a large CDR. Rodent and human CDR sequences are represented by blackened and open boxes, respectively, and the human FRs as solid lines. The maximum size of CDR cloned to date is 19 aa (Cleary et al., 1986). A CDR of this length could be humanised using recombinant PCR primers, X and Y, of 51 nt in length with 15 nt of human FR target sequence complementarity and 36 nt of the rodent CDR sequence. The two oligo sequences would thus overlap by 15 nt, this region of the primary PCR products serving as primers for each other in the recombinant PCR reaction.

(Jones et al., 1986; Queen et al., 1989) have previously been used for the humanisation of antibodies. The above method has benefits over these techniques in that smaller oligos are required in the procedure, even to transfer large CDRs such as the 19 aa CDRH2 present in a number of human IgG subgroup III heavy chains (Cleary et al., 1986). The primary PCR products need only overlap in the middle of the CDR by approx. 15 bp (Higuchi, 1989), and thus to transfer a 57 bp CDR onto the appropriate FR, oligos of a maximum of 51 bp would be required, assuming a homology of 15 bp corresponding to the FR target sequence (Higuchi, 1989; Fig. 5). The above technique is also advantageous over site-directed mutagenesis in that all operations can be performed upon ds DNA without the need for subcloning between ds and ss vectors, thus decreasing the time and effort required to generate the humanised product.

No nt errors were detected in the sequenced clone described. Ho et al. (1989) and Horton et al. (1989), in recombinant PCR mutagenesis studies, have derived error frequencies of $<0.03\%$ and $<0.06\%$, respectively. To further reduce the possibility of nt misincorporation, the template concentration could be increased to 100–1000 ng and the number of PCR cycles reduced (Higuchi, 1989). The C region could also be removed by suitable restriction endonucleases prior to humanisation (Fig. 4), and then subsequently re-ligated to the newly formed, sequenced mosaic V region. This would reduce the size of the final PCR product and thus contribute to a reduced chance of nt misincorporation.

(d) Conclusions

(1) The recombinant PCR technique was used to successfully graft a rat Ig CDR onto a human Ig framework.

(2) No nt misincorporations were found in 1463 bp of sequenced recombinant PCR product.

(3) This procedure should be applicable to the generation of fully humanised mAb nt sequences.

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